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FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
PENN-0794	10/018938

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/40266	21 June 2000	25 June 1999

TITLE OF INVENTION
MOLECULAR CORRELATES OF SCHIZOPHRENIA AND METHODS OF DIAGNOSING SCHIZOPHRENIA VIA THESE MOLECULAR CORRELATES

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

1) Courtesy copy of the International Application;
2) Statement to support filing and submission in accordance with 37 CFR 1.821-1.825;
3) Return post card.

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018938	PCT/US00/40266	PENN-0794

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1040.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$740.00**
☒	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT = **$710.00**

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). **$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	3 - 20 =	0	x $18.00	**$0.00**
Independent claims	3 - 3 =	0	x $84.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS = **$710.00**

☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2. **$355.00**

SUBTOTAL = **$355.00**

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + **$0.00**

TOTAL NATIONAL FEE = **$355.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐ **$0.00**

TOTAL FEES ENCLOSED = **$355.00**

Amount to be: refunded	$
charged	$

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **50-1619** in the amount of **$355.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-1619** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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MOLECULAR CORRELATES OF SCHIZOPHRENIA AND METHODS OF DIAGNOSING SCHIZOPHRENIA VIA THESE MOLECULAR CORRELATES

Field of the Invention

In the present invention, molecular correlates of schizophrenia comprising genes and expressed sequence tags (ESTs) which are differentially regulated in patients suffering from schizophrenia are provided. Using molecular biological procedures which allow precise localization at the single cell level of changes in gene expression within the cortical region, a molecular fingerprint of altered expression of multiple genes in schizophrenia has now been identified. This molecular fingerprint produced from relative levels of mRNAs of genes and ESTs differentially regulated in patients suffering from schizophrenia is useful in the early detection and diagnosis of schizophrenia and in the development and evaluation of agents for the treatment of this disease.

Background of the Invention

The schizophrenic disorders are a group of syndromes manifested by massive disruption of thinking, mood, and overall behavior as well as poor filtering of stimuli. Diagnosis of schizophrenic disorder is currently based upon the presence of a number of behavioral characteristics of at least six months duration including: slowly progressive social withdrawal usually often accompanied by a deterioration in personal care; loss of ego boundaries with the inability to perceive oneself as a separate entity; loose thought associations, often with slowed thinking or overinclusive and rapid shifting from topic to topic; autistic absorption in inner thoughts and frequent sexual or religious preoccupations; auditory hallucinations, often of a derogatory nature; and delusions, frequently of grandiose or persecutory nature. Frequent additional signs

include: flat effect and rapidly alternating mood shift irrespective of circumstances; hypersensitivity to environmental stimuli, with a feeling of enhanced sensory awareness; variability or changeable behavior incongruent with the external environment; concrete thinking with the inability to abstract; inappropriate symbolism; impaired concentration worsened by hallucinations and delusions; and depersonalization, wherein one behaves like a detached observer of one's own actions. Diagnosis of a schizophrenic disorder based upon these behaviors can thus be quite arbitrary and is influenced by sociocultural factors and schools of psychiatric thought. At present, there is no laboratory method for confirmation of a diagnosis of schizophrenia.

Schizophrenic disorders are believed to be of multifactorial cause, with genetic, environmental and neuroendocrine pathophysiologic components. The evidence for significant genetic contribution to schizophrenia is well established. However, the non-mendelian mode of inheritance has made the identification of susceptibility loci challenging (Bowen et al., *Mol. Psychiatry*, 1998, 53(1-2):112-9).

Changes in relative levels of specific brain mRNA species associated with schizophrenia have been disclosed (Perrett et al., *Brain Res. Mol. Brain Res.*, 1992, 12(1-3):163-71). In this study total cellular polyadenylated RNA (poly(A) + RNA, mRNA) was prepared from guanidium thiocyanate extraction of frozen brain tissue from age matched controls and patients suffering from schizophrenia. These mRNA populations were analyzed by *in vitro* translation followed by two-dimensional gel analysis. Relative concentrations of mRNA species coding for four translation products (33 kDa, pI 5.8; 26 kDa, pI 5.8; 35 kDa, pI 7.1; and 23 kDa, pI 6.1) were significantly reduced in schizophrenia compared to controls when determined by

computerized image analysis of fluorograms. Decreased expression of mRNAs encoding two non-N-methyl-D-aspartate receptors, GluR1 and GluR2, in the medial temporal lobe in schizophrenia has also been reported (Eastwood et al., *Brain Res. Mol. Brain Res.*, 1995, 29(2):211-23). Changes in mitochondrial gene expression have also been linked to schizophrenia (Whatley et al., *Neurochem. Res.*, 1996, 21(9):995-1004). In addition, a 3-fold increase in D4 domain receptor mRNA in the frontal cortex of post mortem schizophrenic brain tissue as compared to controls has been reported (Stefanic et al., *Brain Res. Mol. Brain Res.*, 1998, 53(1-2):112-9). However, distinct neurobiological markers that are specific for schizophrenia have remained elusive.

Using molecular biological procedures allowing for precise localization at the single cell level of changes in gene expression, a molecular fingerprint of schizophrenia has now been determined. By identifying altered expression of multiple genes in schizophrenia, methods for early detection and pharmacotherapeutic intervention to alter the course of the disease can be developed.

Summary of the Invention

An object of the present invention it to provide molecular correlates useful in the diagnosis and monitoring of treatment of patients suffering from schizophrenia.

Another object of the present invention is to provide nucleic acid probes useful in the identification of genes differentially regulated in patients with schizophrenia.

Another object of the present invention is to provide a method of diagnosing schizophrenia in a patient which comprises comparing in a cell or tissue of a patient relative levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia with mRNA levels of genes unaltered in schizophrenic patients.

Another object of the present invention is to provide a method of evaluating agents for treatment of a patient suffering from schizophrenia which comprises: measuring in a cell or tissue of a patient levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia; administering to the patient an agent suspected of being a treatment for schizophrenia; re-measuring in a cell or tissue of the patient levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia; and comparing the levels of mRNAs measured before and after administration of the agent to determine whether the agent altered the mRNA levels of the patient.

Detailed Description of the Invention

Schizophrenia is a chronically debilitating psychiatric disease affecting approximately 1% of the general population. In the last twenty years, research into the neurobiological and molecular substrates of schizophrenia has led to the identification of several structural abnormalities in the brains of schizophrenics (Arnold, S.E. and Trojanowski, J.Q., *Acta Neuropathol.*, 1996, 92:217-231; Davis et al., *Bio. Psychiatry*, 1998, 43:783-793), but no lesions specific to schizophrenia have been identified. Several cortical and subcortical regions have been implicated in the pathogenesis of schizophrenia, in particular the temporal lobe, including the hippocampus, subiculum and entorhinal cortex.

The entorhinal cortex, an integral component of the conduit through which information flows to the hippocampus, helps regulate cortical-hippocampal-subcortical interactions. More specifically, stellate cells in Layer II of the entorhinal cortex are integral to the flow of information (Van Hoesen, G.W., *Trends in Neurosci.*, 1992, 5:345-350). Disruption of the functional integrity of these neurons may contribute to the aberrant behaviors

associated with schizophrenia. Various abnormalities of these neurons have been described in neuropathologic studies of schizophrenia, including aberrant cytoarchitectural arrangement (Arnold et al., *Biol. Psychiatry*, 1997, 42:639-647; Arnold et al., *Arch. Gen. Psychiatry*, 1991, 48:625-632; and Jakob, H. and Beckmann, H. J., *Neural Trans.*, 1986, 65:303-326), smaller neuron size (Arnold et al., *Am. J. Psychiatry*, 1995, 152:738-748), decreased expression of the microtubule-associated protein MAP2 (Arnold et al., *Proc. Natl Acad. Sci. USA*, 1991, 88:10850-10854), and altered catecholaminergic and glutamanergic innervation (Akil, M. and Lewis, D.A., *Soc. Neurosci. Abstr.*, 1995, 21:238; Longson et al, *J. Neural Trans*,. 1996, 103:503-507; Eastwood et al., *Mol. Brain Res.*, 1995, 29:211-223). The strategic location and identified biological correlates of this discrete neuronal population make Layer II neurons of the entorhinal cortex an excellent candidate for probing disease-related differences in gene expression.

Identifying neurobiological correlates for psychiatric disorders has been complicated by several factors, including: the heterogeneity of the cortical and subcortical regions, the complexity of the mammalian CNS and the relative insensitivity of existing molecular techniques at the cellular level, which cannot discern changes occurring at the affected or target neuron from those occurring in the pooled neuronal population. However, currently available array methodologies, which have candidate cDNA probe sequences immobilized on a solid support, now allow for the simultaneous assessment of thousands of genes. Compared to other methods they provide a more complete representation of the orchestrated expression of thousands of genes, while measuring the levels of expression of these genes in different tissue samples.

In the present invention, microarray technologies have been combined with single cell gene expression methodologies to successfully assess transcripts that are differentially regulated between schizophrenic and normal states.

To identify transcripts that are differentially expressed in schizophrenics versus age-matched non-psychiatric controls, mRNA expression was first assessed in entorhinal cortex and individual entorhinal cortex Layer II stellate neurons of schizophrenic samples, using both a candidate gene approach and a variety of high density array platforms. The brain tissue used in this study was obtained from the established brain bank of the Mental Health Clinical Research Center on Schizophrenia at the University of Pennsylvania. The prospectively accrued and assessed subjects in this collection may be particularly instructive because they represent the most severe end of the schizophrenia spectrum, having required hospitalization for many years. Brain sections were immunohistochemically stained with a monoclonal antibody to poorly phosphorylated neurofilament protein. Immunoreactivity in the entorhinal cortex, confined to the somatodendritic region of neurons in Layers II/III and V, was used to identify Layer II/III stellate neurons for subsequent dissection.

Following *in situ* transcription using an oligo-dT-T7 oligonucleotide as a primer, individual entorhinal cortex neurons were dissected. The cDNA present in the respective neurons was amplified, using the aRNA amplification procedure, and was labeled for subsequent reverse Northern blotting analysis. aRNA from three neurons from each of three schizophrenic patients and three controls were pooled, respectively, for initial screening of the high-density cDNA array platforms. Arrays were also screened with cDNA made from pooled total RNA isolated from

the entorhinal cortex of two schizophrenic patients and two matched controls. The ratio of hybridization intensities, as visualized from the arrays between the two groups, was used as the dependent measure of differential expression.

From the initial screen, 120 clones that displayed the highest hybridization difference ratios (60 clones over-expressed and 60 under-expressed in the schizophrenic group) were selected as candidate genes for secondary screening. Comparison of the expression levels of various mRNAs from each schizophrenic and control subject revealed a number of transcripts that were differentially regulated, including those corresponding to proteins involved in the neuro-secretory pathway, one of the most highly regulated groups. Scattergrams of mRNAs encoding transcription factors, ion channels, G-protein coupled receptors and components of the secretory pathway demonstrate the degree of differential regulation between schizophrenic and control groups. Among the cDNA classes, transcription factors showed the significant differences in expression between schizophrenic and control groups. Since transcription factors can alter the expression of a myriad of down-stream genes, it is likely that the regulation of transcription for numerous genes is altered in schizophrenics. Due to concern that the pharmacological course of treatment for schizophrenia may influence gene expression, the initial screening of the arrays used brain tissue from patients who had not received antipsychotic medication for a minimum of one year prior to death.

The changes in gene expression, observed from the array analysis, confirm previous results reporting alterations in single transcripts from schizophrenic brains. For example, two recent studies have reported decreased SNAP-25 protein levels in the temporal cortex and terminal regions of entorhinal cortex projections (Thompson et al., *Biol. Psychiatry*, 1998, 43:239-243; Young et al.,

Cerebral Cortex, 1998, 8:261-268). The alterations in the mRNA levels for various proteins that are intimately associated with neuro-secretory processes were specifically examined. For example, syntaxin mRNA was up-regulated (4.4 fold) in schizophrenics, in agreement with previous reports of increased syntaxin protein levels in schizophrenics (Gabriel et al., *Neuroscience*, 1997, 78:99-110). Several mRNAs encoding neuro-secretory proteins also were differentially regulated between schizophrenia and controls, including down-regulation in schizophrenia of including: SNAP-25 (4.4 fold), γ -adaptin (5.5 fold), synaptic vesicle amine transporter (3.5 fold), synaptotagmin 1 (3.1 fold), synaptotagmin IV (2.5 fold), GABA transporter 1 (1.7 fold), synaptophysin (1.4 fold), noradrenaline transporter (0.8 fold), and synaptotagmin V (0.7 fold). The proteins encoded by these mRNAs are associated with several sequential phases of neuro-secretory processes: loading of vesicles with neurotransmitter (transporters); docking of the vesicles with the membrane (synaptotagmin, SNAP-25 and syntaxin); and, finally, vesicle recycling (γ-adaptin) (Sudhof, T.C. Nature 1995 375:645-653). These data indicate that pre-exocytotic steps, endocytosis and recycling are dysregulated in the enterohinal cortex of schizophrenics. This dysregulation could lead to dysfunctional neurotransmission without structural neuropathological consequences. An increase in neurotransmitters in the synaptic space could thus occur, thereby increasing presynaptic stimulation. Taken together, these results indicate that the dysregulation of specific components of neuro-secretory/ neurotransmitter pathways may be the mechanism of the neuronal dysfunction underlying schizophrenia.

Differential hybridization to the cDNAs encoding several expressed sequence tags (ESTs) was also noted, in

addition to genes of known function. ESTs are markers for human genes actually transcribed *in vivo* and comprise DNA sequences corresponding to a portion of nuclear encoded messenger RNA. The ESTs relating to the present invention generally represent relatively small coding regions or untranslated regions of human genes. Although most of these sequences do not code for a complete gene product, the ESTs are highly specific markers for the corresponding complete coding regions. The ESTs are of sufficient length that they will hybridize, under stringent conditions, for example, where at least 95% identity (base pairing) is required for hybridization. The property permits use of the identified ESTs to isolate the entire coding region and even the entire sequence of additional genes differentially regulated in schizophrenia.

Thus, each of the ESTs corresponds to a particular unique human gene. Knowledge of the EST sequence permits routine isolation and sequencing of the complete coding sequence of the corresponding gene. The complete coding sequence is present in a full length cDNA clone as well as in the gene carried on genomic clones. Therefore, each EST corresponds to a cDNA (from which the EST was derived), a complete genomic gene sequence, a polypeptide coding region and a polypeptide or amino acid sequence encoded by that region. Accordingly, these ESTs can be expanded to provide the full coding regions thus making it possible to identify previously unknown genes differentially regulated in individuals suffering from schizophrenia.

Several of the most highly regulated ESTs were sequenced, one of which corresponded to phospholemman (PLM), a phosphoprotein involved in the formation and/or regulation of a CI anion channel. PLM mRNA and protein are enriched in cardiac and skeletal muscle, although Northern analysis has demonstrated moderate mRNA expression in total brain homogenates (Chen et al., *Genomics*, 1997, 41:435-

443). PLM mRNA expression levels in single enterohinal cortex stellate neurons were lower in schizophrenic brains than in matched controls. To determine whether phospholemman protein is present in Layer II/III stellate neurons, a polyclonal antibody against PLM protein was used to stain sections adjacent to those used for neuronal dissection and mRNA analysis. PLM immunoreactivity was detected in two distinct cellular compartments in the human brains. A similar distribution was observed in rat brains. Diffuse, cytoplasmic PLM immunoreactivity was detected within the perikarya of entorhinal cortex stellate neurons and neocortical pyramidal cells, and punctate PLM-immunoreactivity was found in preterminal axons and terminal fields throughout the hippocampal formation. Perforant pathway labeling was particularly distinct. Semi-quantitative assessment of the 24 cases, by experimenters "blind" to the diagnosis, revealed differences in cytoplasmic PLM immunoreactivity within the perikarya of Layer II enterohinal cortex stellate neurons. Specifically, perikaryal PLM immunoreactivity in enterohinal cortex stellate neurons was consistently less intense in the schizophrenic brains than in the normal control brains. No differences were observed in the intense axonal/terminal labeling of the perforant path axons that traverse the subicular complex and terminate within the dentate gyrus.

Based on these data it is believed that PLM may have two possible functions in these neurons. First, based on the role of PLM in anion channel conductance in Xenopus oocytes and lipid bilayers (Moorman et al., *Circulation Res.*, 1998, 82:367-374), it is believed that decreased expression of PLM mRNA and protein levels may shift enterohinal cortex stellate neurons in schizophrenic patients into an altered electrophysiological state. The magnitude of such alterations would be dependent on the

role of PLM in maintaining the CI gradient in these neurons. Alternatively, these observations are consistent with PLM being a functional anion channel associated with the secretory vesicles. In this capacity, PLM modulates the ionic balance in the vesicle, thereby altering the properties of these vesicles and, possibly, any post-translational processing of proteins that may occur within these vesicles. Moreover, the pattern of PLM immuno-staining is distinct from that obtained for syntaxin, where there is clear cell perikaryal and axonal staining. Since syntaxin is associated with secretory vesicles these data suggest that in schizophrenia, PLM may be present in a subclass of secretory vesicles in these neurons. The observed enterohinal cortex staining pattern is not selective to this brain collection population; it was replicated in enterohinal cortex tissue sections from two schizophrenic patients obtained from the Stanley Foundation Brain Bank.

In addition to mRNAs associated with neuro-secretory mechanisms, differential expression of mRNAs that had previously been examined in different schizophrenic populations, including various glutamate and nicotinic receptor subunit mRNAs, was observed. Previous studies have indicated alterations in glutamanergic activity, including decreased KA binding sites (Kerwin et al. Neuroscience 1991 39:25-32) and decreased abundance of GluRI mRNA (Harrison et al., *Lancet*, 1991, 337:450-452), KA2 and GluR6 mRNAs in the hippocampus (Porter et al., *Brain Res.*, 1997, 751:217-231). More recently, decreased expression of GluR2 mRNA in the parahippocampal gyrus, including the EC18 and increased flip/flop ratios of GluR2 in the hippocampus of schizophrenics has been found (Eastwood et al., *Mol. Brain Res.*, 1997, 44:92-98). In experiments described herein, a decrease in NMDAR1-2A subunit (1.6 fold), GluR2 (1.5 fold) and GluRl (1.7 fold)

was observed, with no apparent change in mRNA abundances for GluR6 or NMDA-NR1. Cholinergic dysfunction has also been implicated in schizophrenia, including decreased nicotinic receptor binding in the hippocampus (Freedman et al., *Biol. Psychiatry*, 1995, 38:22-33) and the demonstration of a dinucleotide polymorphism, at chromosome 15q13-14, the site of the α7 subunit of the nicotinic receptor (Freedman et al., *Proc. natl Acad. Sci. USA*, 1997, 94:587-592). In experiments described herein, a 2.7 fold decrease in expression of the α7 subunit mRNA was observed in stellate neurons of the entorhinal cortex of the schizophrenic population examined. Taken together, these data indicate that impaired entorhinal cortex function may occur in schizophrenia due to glutamanergic mRNA expression. The ability to examine coordinate changes or ratios of receptor subunit mRNAs yields insight into differential expression of receptor-heteromer composition in schizophrenia.

There have been many reported genetic linkages for schizophrenia which appear to be family specific. Possible explanations for this include improper grouping of affected individuals. Also, distinguishing the clinical features of schizophrenia from other psychotic diseases can be difficult. Cohort designation is critically important to the proper genetic and biochemical analysis of schizophrenia. Additionally, since schizophrenia appears to be a multigenic disease, it is likely that alterations in the orchestrated expression of multiple genes contribute to the disease. Approximately 25% of the genes in the public databases have been mapped to chromosomal loci. Using this information, the relative abundances of various mRNAs whose genes map to presumed schizophrenia linkage sites have been examined. Results from this comparison are depicted in Table I. Data are presented as the number of genes screened which exhibit the designated change.

Table I

Linkage Site	Schizophrenic>Normal				Normal
	4X>	2-4X	1.5-2X	No change	1-5-2X
5q11		6	7	28	3
5p14	1		1	9	1
6p22-23	2	8	5	35	3
8p21-22		3	2	26	1
10q21-22	2	17	15	58	8
13q14		4	4	22	4
13q32	2	6	6	28	2
15q13-14		2		14	1
22q12	2	18	12	69	15
Xq24	2	10	13	41	8

The GenBank Accession number for those mRNAs for which the abundances change and map to these sites are: 5q11-S/N 1.5-2 (T65606, R14837, R01976, T78213, H17693, H12917, H56735), 2-4(N40834, AA040100, N91733, R20850, N36349, AA069027), 4-8(0), N/S 1.5-2(H90997, T52078, AA181981), 2-4(AA134752),4-8(0); 5p14-S/N 1.5-2(R52325),2-4(0),4-8(T97193), N/S 1.5-2(R33908), 2-4(R33908), 4-8(0); 6p22-23-S/N 1.5-2(R13822, R18757, R67103, N39825, N90967), 2-4(R35429, R12852, R73377, H80035, H06471, R59686, T93822, AA063104), 4-8(R55914, R55914), N/S 1.5-2(H05555, W88585, R75967), 2-4(R20393), 4-8(0); 8p21-22-S/N 1.5-2(N48138, H50016), 2-4(AA057722, T97031, AA065205), 4-8(0), N/S 1.5-2 (AA176162), 2-4(H86379, R10016, AA147552), 4-8 (0); 10q21-22-S/N 1.5-2(H18544, H18580, H86374, T94968, R36505, N58146, R66021, H23362, R35367, AA041317, R86895, R20902, H88208, H96049, H95817), 2-4(T97973, N94199, AA034359, W89028, AA031547, T94513, W33161, N50000, AA056151, N73236,

R13021, T92992, R73467, AA150316, W24565, R99739, N77156), 4-8(T92520, R83083), N/S 1.5-2 (T92520, R83083, T92520, R83083, T92520, R83083, T92520, R83083), 2-4(N45679, AA130293, AA204895, AA197156, N31404, H03532, T90167, H09945, H43746), 4-8(0); 13q14-S/N 1.5-2(R83060, AA042832, R13574, W30787), 2-4(N79903, W37952, AA098909), 4-8(0), N/S 1.5-2(R64064, H00263, W04203, N75196), 2-4(R28187, W16727, W17249), 4-8(0); 15q13-14-S/N 1.5-2(0), 2-4(W03952, R12985), 4-8(0), N/S 1.5-2(H58462), 2-4(H03759), 4-8(0); 22q12-S/N 1.5-2(N40124, R97618, H72550, R85629, R92856, N76363, AA063107, W01484, T74008, H20677, H19770, AA057038), 2-4(H72029, R72020, R56380, H38478, H19245, R54671, H15212, H24175, R51454, AA076650, T70749, AA029590, W25194, R13055, T89772, R10794, AA010608), 4-8(AA046862, N59753), N/S 1.5-2(T68427, R10652, H14385, H62176, R69153, R22532, R18967, H67332, W51822, AA056636, H73348, H12952, W46211, R23382, AA205659), 2-4(R22377, W47243, W37799, W32354, H58182, N30964, N47247), 4-8(AA214079); Xq24-S/N 1.5-2(H13007, H92239, N35752, R63553, N99032, H87640, W04972, T96195, R17860, R26624, R35360, R35360, N94781), 2-4(AA034404, N58691, AA058497, N59049, R11244, R36437, AA113044, T66128, H52441, R35826), 4-8(R35028, T51728), N/S 1.5-2(N27567, H43560, W16945, R23654, AA053212, N32388, N34591, AA134026), 2-4(N57166, W31672, AA149353, T99984, W49540, N24481, H89195, W38961), 4-8(R35028, T51728).

From this analysis, it is clear that the abundances of most of these mRNAs remains relatively unchanged within these regions. However, some show dramatic differences. Individually, these particular mRNAs are unlikely to be key causative factors of schizophrenia, yet small changes in multiple genes spanning these different chromosomal sites may indeed result in an altered cellular physiology and contribute to the schizophrenic phenotype.

The ability to evaluate the prevalence of a substantial portion of genes in the human genome at the

level of the single cell provides a more complete transcriptor of the affected neuronal populations in schizophrenia. In turn the transcriptor can be used to identify or define pharmacological targets for the treatment of the disease. Assessing changes at the level of the single cell is of particular importance when assessing gene expression in heterogeneous neuronal populations such as the enterohinal cortex. Reliance on regional assessment of gene expression emphasizes the genes contained in the majority of the neuronal population and/or those in highest abundance in the region, which may not adequately reflect alterations in gene expression in target neurons. Moreover, changes in gene expression occurring in the target neurons may be masked by the changes in the pooled neuronal population. In order to account for such differences, changes in gene expression in the enterohinal cortex and in Layer II/III stellate neurons from the enterohinal cortex were examined. The hybridization patterns for a subset of 96 genes from a Synteni GEM array were determined for stellate neurons and enterohinal cortex tissue samples from schizophrenics and controls. Differential expression of a number of genes was observed between schizophrenia and controls for both the enterohinal cortex and pooled stellate neurons. In addition, several genes that were expressed in the pooled stellate neurons were also expressed in the enterohinal cortex at differing abundances. Indeed some mRNAs were only detectable in the pooled neurons and not in the enterohinal cortex likely because they are enriched in the examined neuronal population. Also many mRNAs were present in the enterohinal cortex sample that were not detectable in the pooled neurons likely due to the heterogeneity of cell type in the enterohinal cortex samples and the consequent dilution effect. These results indicate that, when targeting neurons in heterogeneous neuronal populations for

analysis, it is informative to define the level of molecular analysis to the single cell as well as to analyze the tissue sample.

Thus, screening of over 30,000 cDNAs via this method has now led to the identification of multiple genes and ESTs that are differentially regulated in individuals diagnosed with schizophrenia compared with age-matched controls. Examples of these molecular correlates for schizophrenia include, but are not limited to, mRNA for γ -adaptin, synaptic vesicle amine transporter, synaptotagmin 1, synaptotagmin IV, GABA transporter 1, synaptophysin, noradrenaline transporter, synaptotagmin V, phospholemman (PLM), NMDAR1-2A subunit, GluR2, GluR1, and α7 subunit.

The relative levels of each of the mRNAs of the identified genes or ESTs to each other or a subset of this group or the whole group is diagnostic of schizophrenia. By "relative levels" it is meant either that the level of a selected mRNA or multiple mRNAs in a patient is compared to other mRNA levels in the same patient or that the level of a selected mRNA or multiple mRNAs in a patient is compared to levels of the same mRNA or mRNAs in healthy individuals. Thus, in this method, relative levels of mRNAs of the identified genes and ESTs determined in a cell or tissue of a patient suspected of suffering from schizophrenia are compared with relative levels of mRNAs which are not altered in schizophrenia in the cell or tissue of the same patient. Alterations in mRNA levels of the identified genes and ESTs as compared to other mRNA levels in the cell or tissue of the same patient are indicative of schizophrenia. By "alterations" it is meant an increase or decrease in the relative level of a selected mRNA or group of mRNAs of the identified genes or ESTs.

It is believed that relative changes in expression of the identified genes and/or ESTs are also useful in identifying the molecular phenotype of this schizophrenic

disease state. Identifying the molecular phenotype of a schizophrenic patients is useful in prognosticating the success of various treatments for this patient.

Further it is believed that regulation of mRNA levels of the identified genes and ESTs through therapeutic agents may be useful in the treatment of schizophrenia. Accordingly, the identified genes and ESTS may be useful in developing new therapeutic agents for the treatment of this debilitating disease. Further, monitoring of mRNA levels of the identified genes and ESTS may be useful in assessing the therapeutic value of new agents for treatment of schizophrenia.

The following nonlimiting examples are provided to further illustrate the present invention.

EXAMPLES

Example 1: Subjects

Brains from 10 chronically hospitalized patients with schizophrenia and 10 age-matched neurologically normal controls were used. Schizophrenic subjects were from elderly, "poor-outcome" patients who were participants in a clinicopathological studies program at the University of Pennsylvania in collaboration with eight state hospitals in eastern and central Pennsylvania. All patients were prospectively accrued, had clinical interviews and assessment, and were diagnosed according to DSM-IV1 criteria by research psychiatrists of the Mental Health Clinical Research Center. In general, clinical features, included prominent negative symptoms, relatively mild positive symptoms, moderate to severe cognitive dysfunction, and impairments in basic self-care activities that warranted their chronic hospitalization.

Control subjects were obtained via the Center for Neurodegenerative Disease Research at the University of Pennsylvania. They were without history of neurologic or major psychiatric illness.

Gross and microscopic diagnostic neuropathologic examinations, which included examination of multiple cortical and subcortical regions, were performed in all cases, and no neuropathological abnormalities relevant to mental status were found.

Example 2: Histochemistry

Tissue blocks, which included the middle portion of the entorhinal cortex, were dissected from the ventromedial temporal lobe at autopsy, fixed in ethanol (70%/150 mM NaCl) and embedded in paraffin. Sections (6 μm) were mounted on microscope slides previously coated with chrom alum (0.25%). Prior to manipulation, sections were de-paraffinized and re-hydrated (xylene-100% ethanol-95% ethanol-80% ethanol-70% ethanol). One section from each individual was stained with acridine orange to assess the presence of nucleic acids in the sections. Following verification of the presence of nucleic acids in the tissue sections, additional sections containing the enterohinal cortex were immunolabeled with a mouse monoclonal antibody to mid-sized, poorly phosphorylated neurofilament (RMDo20) in 0.1 M Tris/2% denatured horse serum overnight at 4°C. The antibody was labeled with the avidin-biotin method (ABC Vectastain, Vector Laboratories, Burlingame, CA) and visualized with 3,3'-diaminobenzidine (DAB). For phospholemman, tissue sections were pretreated. with methanol and hydrogen peroxide prior to the addition of polyclonal anti-phospholemman(1: 1,000 dilution) in 2% DHS in Tris buffer (pH 7.4) and development, using the ABC kit (Vector Laboratories).

Example 3: *In situ* transcription and aRNA amplification

Following immunolabeling with RMDO-20, an oligo(dT)-T7 primer/promoter [AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGG CGC(T)24 (SEQ ID NO: 1)] was hybridized to poly(A+)mRNA on the immunohistochemically stained sections overnight in 50% formamide/5X SSC. Complimentary DNA (cDNA) was synthesized

(via *in situ* transcription as described by VanGelder et al. Proc. Natl Acad. Sci. USA 1990 87:1663-1667) using avian myeloblastosis virus reverse transcriptase (AMVRT, 0.5 U/λ, Seigagaku America, USA) in Tris buffer containing 6 mM $MgCl_2$, 120 mM KCl, 7 mM dithiothreitol, 250 μM each of dATP, dCTP, dGTP and TTP, and 0.12 U/λ of RNAsin. Following morphological identification of entorhinal cortex stellate neurons in Layer II/III, cell bodies were dissected using a micropipette attached to a micromanipulator under low power objective field (40X) with minimal disruption of surrounding neuropil. Contents were collected in the pipette and emptied into 1.5 ml microcentrifuge tubes for second strand cDNA synthesis and subsequent aRNA amplification. Amplification and re-amplification procedures were conducted in accordance with procedures described by Eberwine et al. Proc. Natl Acad. Sci. USA 1992 89:3010-3014. Two rounds of aRNA amplification result in approximately a 10^6 fold increase over the original amount of transcript in the cell.

Example 4: cDNA Arrays

^{32}P-labelled cDNA or aRNA was used to probe various array platforms from Research Genetics (-7000 genes) and Genome Systems (Human Gene Discovery Arrays; >18,000 genes). Fluorescent-labeled probes were used to screen the Synteni high density arrays (Gems; >10,000 genes). Following initial screening, selected cDNAs were linearized, slot blotted on NYTRAN net neutral charge nylon transfer membrane (Schleicher and Schuell, Keene, NH) using slot blot apparatus (Millipore Corp., Bedford, MA), and probed with ^{32}P-labelled aRNA. DNA was crosslinked to the membrane by ultraviolet irradiation or baking at 85°C overnight under vacuum. The Genome Systems arrays and membranes for the secondary screens were hybridized for 48 hours at 44°C in a rotisserie hybridization oven with the following hybridization solution: 50% formamide (Fluka

Ultrapure, Fluka Chemical Co., Ronkonkoms, NY), 5X SSC, 5X Denhardt's solution, 0.1% sodium dodecyl sulfate, 200 ng of sheared salmon sperm, and 1.0 mM sodium pyrophosphate. Arrays were washed sequentially with 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS and 0.1X SSC/0.1% SDS for 20 minutes each at 44°C. Labeled hybridized products were detected using phosphoimager cassettes and Image StormScanner (Molecular Dynamics, Sunnyvale, CA).

Example 5: Data Analysis/Relational Database

The Genome Systems data were imported into RAD, a Sybase relational database developed at the University of Pennsylvania. RAD was designed to capture information on RNA abundance assays for any type of cDNA filter or microarray platform. For each experiment, the intensities for data points were expressed as a percentage of the total intensity. This enabled comparison of data generated under different conditions and experimental platforms. To identify genes by functional role or chromosomal location, queries were performed against DOTS (Database of Transcribed Sequences), a Sybase relational database also developed at the University of Pennsylvania. DOTS contains known and putative transcripts from human and mouse. Each transcript has a consensus sequence assembled by computational analysis of the EST and known mRNA sequences available in the public databases. Cellular roles were assigned to transcripts in DOTS with high sequence identity to the set of experimentally characterized mRNAs described and annotated in the EGAD database. DOTS transcripts were assigned chromosomal locations if their consensus sequences contained an EST sequence that had been mapped in the GeneBridge4 radiation-hybrid mapping panel. Clones arrayed on the GenomeSystems filters or used as the source of DNA for the PCR product on the Synteni microarrays are derived from the I.M.A.G.E. clone set, and can be linked to the DOTS transcripts through the EST sequences. This allowed

assignment of cellular role to 2,672 and chromosomal location to 11,591 GenomeSystems spots. Data sets were selected by SQL queries spanning the DOTS and RAD databases, and scatter plots generated using Microsoft Excel.

What is Claimed is:

1. A molecular correlate diagnostic of schizophrenia comprising a nucleic acid encoding γ -adaptin, synaptic vesicle amine transporter, synaptotagmin 1, synaptotagmin IV, GABA transporter 1, synaptophysin, noradrenaline transporter, synaptotagmin V, phospholemman, NMDAR1-2A subunit, GluR2, GluR1, or α7 subunit.

2. A method of diagnosing schizophrenia in a patient comprising comparing in a cell or tissue of a patient relative levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia with mRNA levels of genes unaltered in schizophrenic patients.

3. A method of evaluating agents for treatment of a patient suffering from schizophrenia comprising:

(a) measuring in a cell or tissue of a patient levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia;

(b) administering to the patient an agent suspected of being a treatment for schizophrenia;

(c) re-measuring in a cell or tissue of the patient levels of mRNAs for genes and expressed sequence tags differentially regulated in patients suffering from schizophrenia; and

(d) comparing the levels of mRNAs measured in the cell or tissue before and after administration of the agent to determine whether the agent altered the mRNA levels of the patient.

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(54) Title: MOLECULAR CORRELATES OF SCHIZOPHRENIA AND METHODS OF DIAGNOSING SCHIZOPHRENIA VIA THESE MOLECULAR CORRELATES

(57) Abstract: Molecular correlates of genes and expressed sequence tags useful in the diagnosis and monitoring of treatment of patients suffering from schizophrenia are provided.

SEQUENCE LISTING

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<120> Molecular Correlates of Schizophrenia and Methods of Diagnosing Schizophrenia via This Molecular Correlates

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Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Molecular Correlates of Schizopherenia and Methods of Diagnosing Schizophrenia via these Molecular Correlates

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **21 June 2000** as United States Application No. or PCT International Application Number **PCT/US00/40266**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/141,160	June 25, 1999
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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26259

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